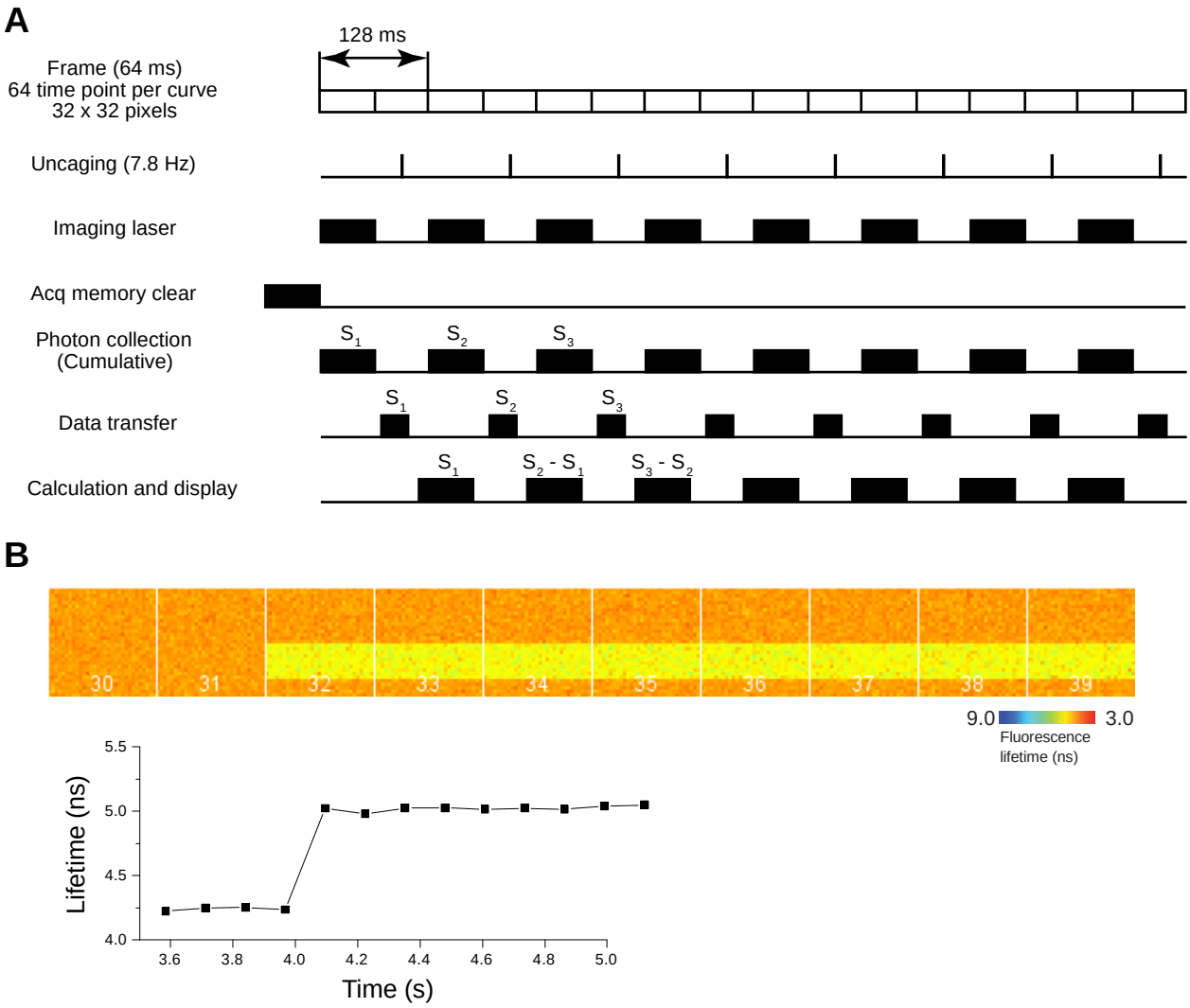

Figure S1. Related to Figure 1

Schematic timing of fast-frame FLIM data acquisition and uncaging

(A) We collected photons cumulatively throughout an imaging session (typically 768-1536 frames), since the memory clearance is relatively time consuming. The photon collection and data transfer were done alternatively in every other frame. Following the data transfer, the image was processed and displayed. The number of photons acquired in the previous frame was calculated as the data transferred in the current frame subtracted by that done two frames ago. When we performed uncaging, uncaging pulses were applied in the frame in which photons were not collected. The imaging laser was turned off when photons were not collected. The acquisition software was written in MatLab. Since the number of photons per pixel per lifetime channel never exceeded 10 photons, the signal did not saturate the memory (16 bits) during data acquisition.

(B) The temporal resolution of the system. Top: fluorescence lifetime images of fluorescein solution (1 μM) acquired at 128 ms/frame (laser tuned at 920 nm). The number denotes the frame number of images. Pulses of another 720 nm laser was applied during imaging at 7.8 Hz for 20 ms (= 10 lines) every frame from the frame #32. Because the 720 nm laser is not synchronized with photon counting, it artificially increases the measured fluorescence lifetime (appears as yellow bar in the lifetime images). The sharp edge at the uncaged time indicates that the temporal resolution is higher than 1 line (2 ms). Bottom: the time course of fluorescence lifetime in the lines corresponding to uncaging time.

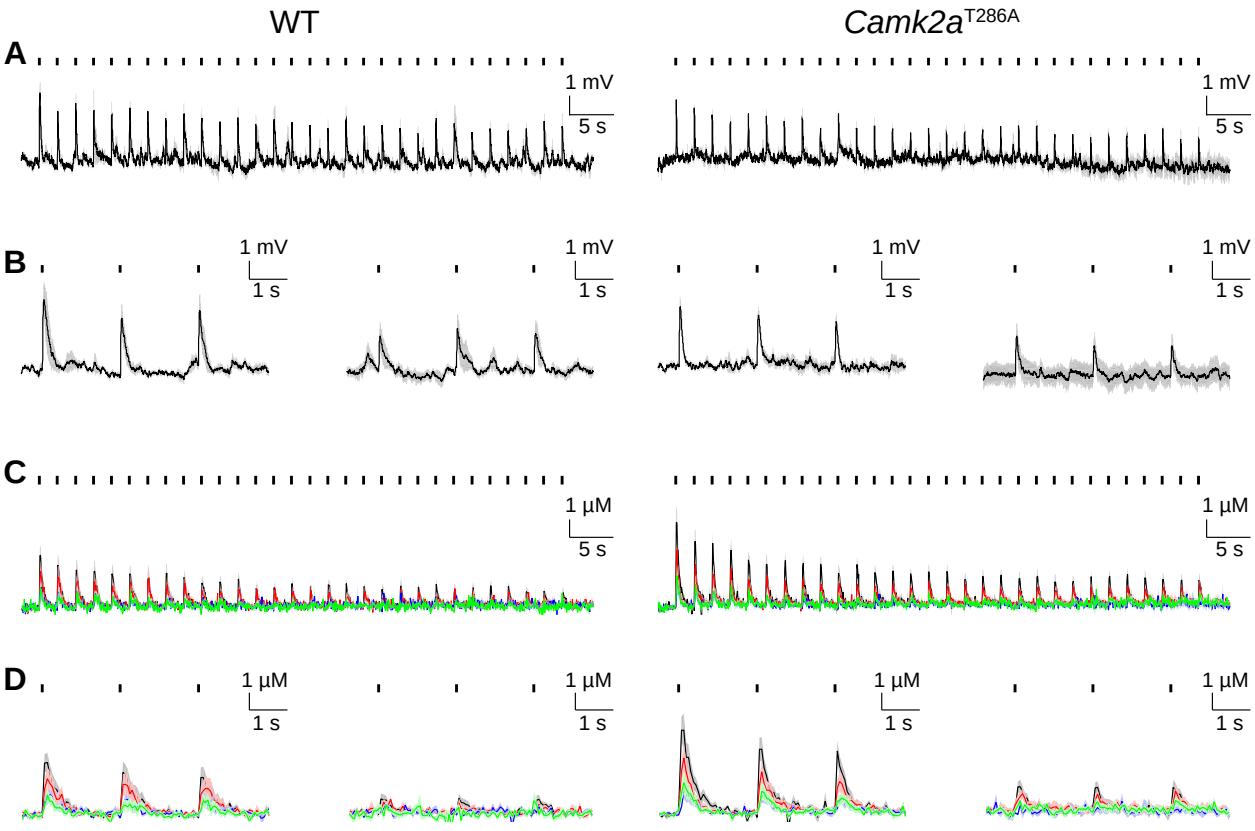

Figure S2. Related to Figure 1

Measurements of uncaging-evoked EPSCs (uEPSCs) and Ca^{2+} transients during glutamate uncaging

(A) Uncaging-evoked EPSCs in CA1 neurons from *Camk2a*^{T286A} mice (right; n = 62 spines/14 neurons) and their wild-type littermates (left; n = 43 spines/9 neurons).

(B) Expanded view of the first and last three uEPSCs shown in (A).

(C) Ca^{2+} transients measured in the stimulated spine (black), adjacent spine (< 3 µm, green), dendritic shaft near (red; < 1 µm) and distant (blue; 1-3 µm) from the stimulated spine during glutamate uncaging. The decreases in the magnitude of Ca^{2+} transients over repetitive uncaging pulses have been reported before (Lee et al. 2009).

(D) Expanded view of the first and last three Ca^{2+} transients shown in (C). All data are shown in mean ± s.e.m.

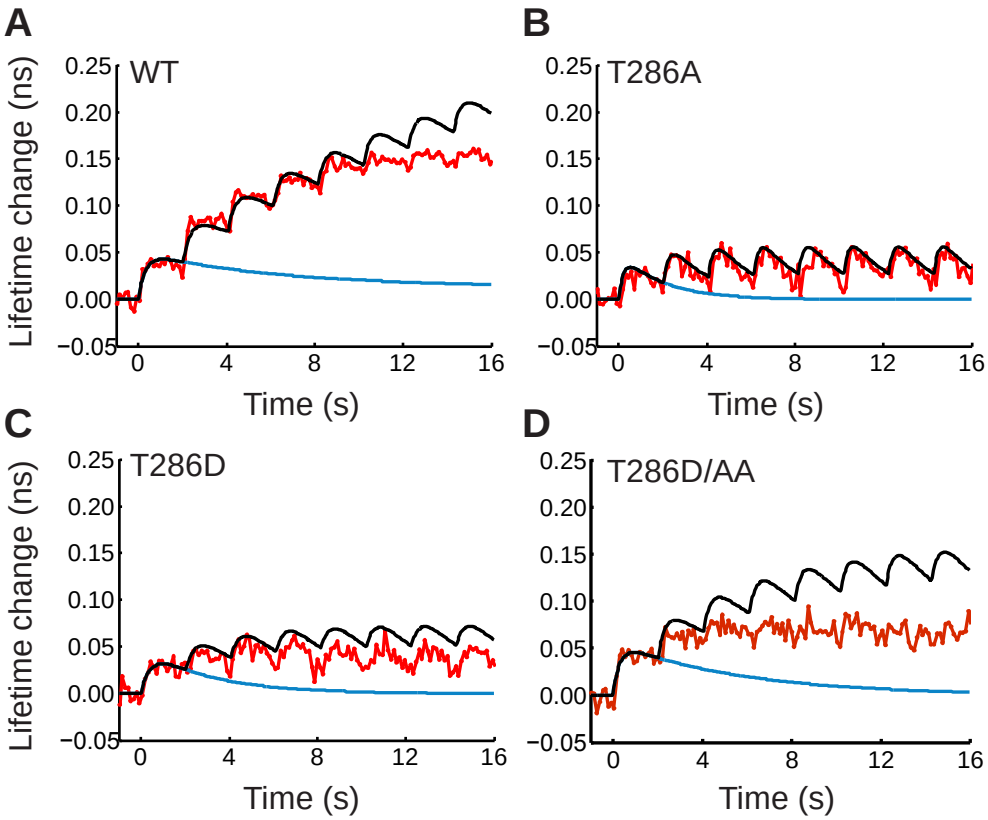

Figure S3. Related to Figure 1 and Figure 2

Model of CaMKII integration property

(A-D) Black: simulated curves of linear summation of fluorescence lifetime change of Camuiα^{WT} (A), Camuiα^{T286A} (B), Camuiα^{T286D} (C), or Camuiα$^{T286D/T305A/T306A}$ (D) in response to 8 pulses of glutamate uncaging at 0.49 Hz. Blue: simulated curves in response to a single glutamate uncaging pulse. Red: fluorescence lifetime change during glutamate uncaging at 0.49 Hz.

(A) A simulated curve based on a function: $F(t) = \left[a \cdot e^{-t/\tau_{fast}} + b \cdot e^{-t/\tau_{slow}}\right] \cdot \left[1 - e^{-t/\tau_{rise}}\right]$, where $a = 0.03, \tau_{fast} = 5.5$, $b = 0.02, \tau_{slow} = 62.5, \tau_{rise} = 0.3$. The parameters are adapted from curve fitting to fluorescence lifetime change of Camuiα^{WT} in response to a single glutamate uncaging pulse to the same function.

(B) A simulated curve is based on a function: $F(t) = C \cdot \left[1 - e^{-t/\tau_{rise}}\right] \cdot e^{-t/\tau_{decay}}$, where $C = 0.05, \tau_{rise} = 0.3, \tau_{decay} = 1.9$. Parameters are adapted from curve fitting to fluorescence lifetime change of Camuiα^{T286A} in response to a single glutamate uncaging pulse.

(C) A simulated curve based on a function: $F(t) = C \cdot \left[1 - e^{-t/\tau_{rise}}\right] \cdot e^{-t/\tau_{decay}}$, where $C = 0.05, \tau_{rise} = 0.3, \tau_{decay} = 3.0$. τ_{decay} is adapted from curve fitting to fluorescence lifetime change of Camuiα^{T286D} in response to 0.49 Hz glutamate uncaging pulse, and C and τ_{rise} are adapted from Camuiα^{T286A} in response to a single glutamate uncaging.

(D) A simulated curve based on a function: $F(t) = C \cdot \left[1 - e^{-t/\tau_{rise}}\right] \cdot e^{-t/\tau_{decay}}$, where $C = 0.05, \tau_{rise} = 0.3, \tau_{decay} = 5.7$. τ_{decay} is adapted from curve fitting to fluorescence lifetime change of Camuiα$^{T286D/T305A/T306A}$ in response to 0.49 Hz glutamate uncaging pulse, and C and τ_{rise} are adapted from Camuiα^{T286A} in response to a single glutamate uncaging.

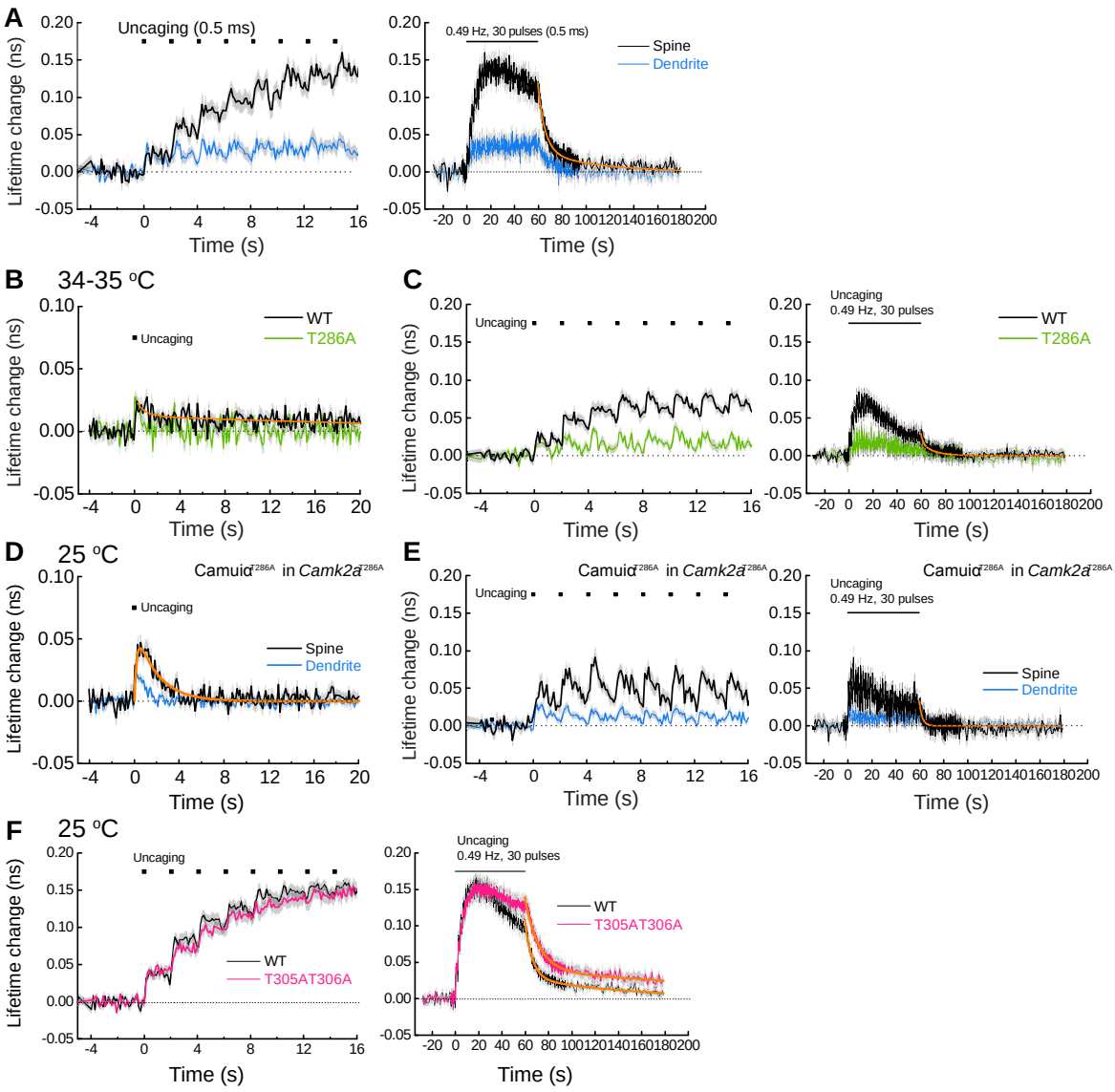

Figure S4. Related to Figure 1 and Figure 2

CaMKII activation under various conditions

(A) CaMKII activation induced by short duration of glutamate uncaging. Averaged change in fluorescence lifetime of Camuiα^{WT} in the stimulated spine (black) and in dendritic region (blue) in response to glutamate uncaging (0.5 ms duration, 8 mW) at 0.49 Hz for 30 pulses. Left panel is expanded view of the right panel. The orange curve on Camuiα^{WT} (right panel) is obtained by curve fitting of a double-exponential function. The decay time constants are obtained as τ_{fast} = 5.8 ± 1.2 s (76%) and τ_{slow} = 46.5 ± 41.9 s (24%) (n =23 spines/5 neurons).

(B-C) Camuiα activation at a near physiological temperature (34-35 °C)

(B) Averaged change in fluorescence lifetime of Camuiα^{WT} (black, n =26 spines/7 neurons) and Camuiα^{T286A} (green, n = 23 spines/6 neurons) in the stimulated spine in response to a single glutamate uncaging pulse. The orange curve on Camuiα^{WT} is obtained by curve fitting of a double-exponential function: $F(t) = F_0 \cdot \left[P_{fast} \cdot e^{-t/\tau_{fast}} + P_{slow} \cdot e^{-t/\tau_{slow}}\right]$. The decay time constants are obtained as τ_{fast} = 0.4 s (57%) and τ_{slow} = 28.1 s (43%).

(C) Glutamate uncaging at 0.49 Hz. Left panel is expanded view of the right panel. The orange curve on Camuiα^{WT} (black) is obtained by curve fitting of a double-exponential function, τ_{fast} = 1.8 ± 1.6 s (45%) and τ_{slow} = 11.0 ± 19.0 s (55%) (n =28 spines/9 neurons). Time course of Camuiα^{T286A} is shown in green (n =19 spines/6 neurons).

(D-E) Activation of Camuiα^{T286A} measured in hippocampal slices from *Camk2a*^{T286A} knock-in mice

(D) Camuiα^{T286A} activation in response to a single glutamate uncaging pulse. The orange curve is obtained by curve fitting of a function: $F(t) = C \cdot \left[1 - e^{-t/\tau_{rise}}\right] \cdot e^{-t/\tau_{decay}}$ where τ_{rise} is fixed during curve fitting (τ_{rise} = 0.3 s). The decay time constant is τ_{decay} = 1.9 ± 0.2 s (n = 37 spines/9 neurons).

(E) Glutamate uncaging at 0.49 Hz. Left panel is expanded view of the right panel. The orange curve is obtained by curve fitting of a function: $F(t) = C \cdot e^{-t/\tau_{decay}}$. The time constant is τ_{decay} = 2.4 ± 1.4 s (n = 17 spines/9 neurons).

(F) Averaged change in fluorescence lifetime of Camuiα$^{T305A/T306A}$ (red, n = 26 spines/4 neurons) in the stimulated spine during glutamate uncaging. The decay time constants are obtained as τ_{fast} = 11.8 ± 0.5 s (72%) and τ_{slow} = 255 ± 153 s (28%). All data are shown in mean ± s.e.m, and s.e.m of time constants is obtained by bootstrapping.

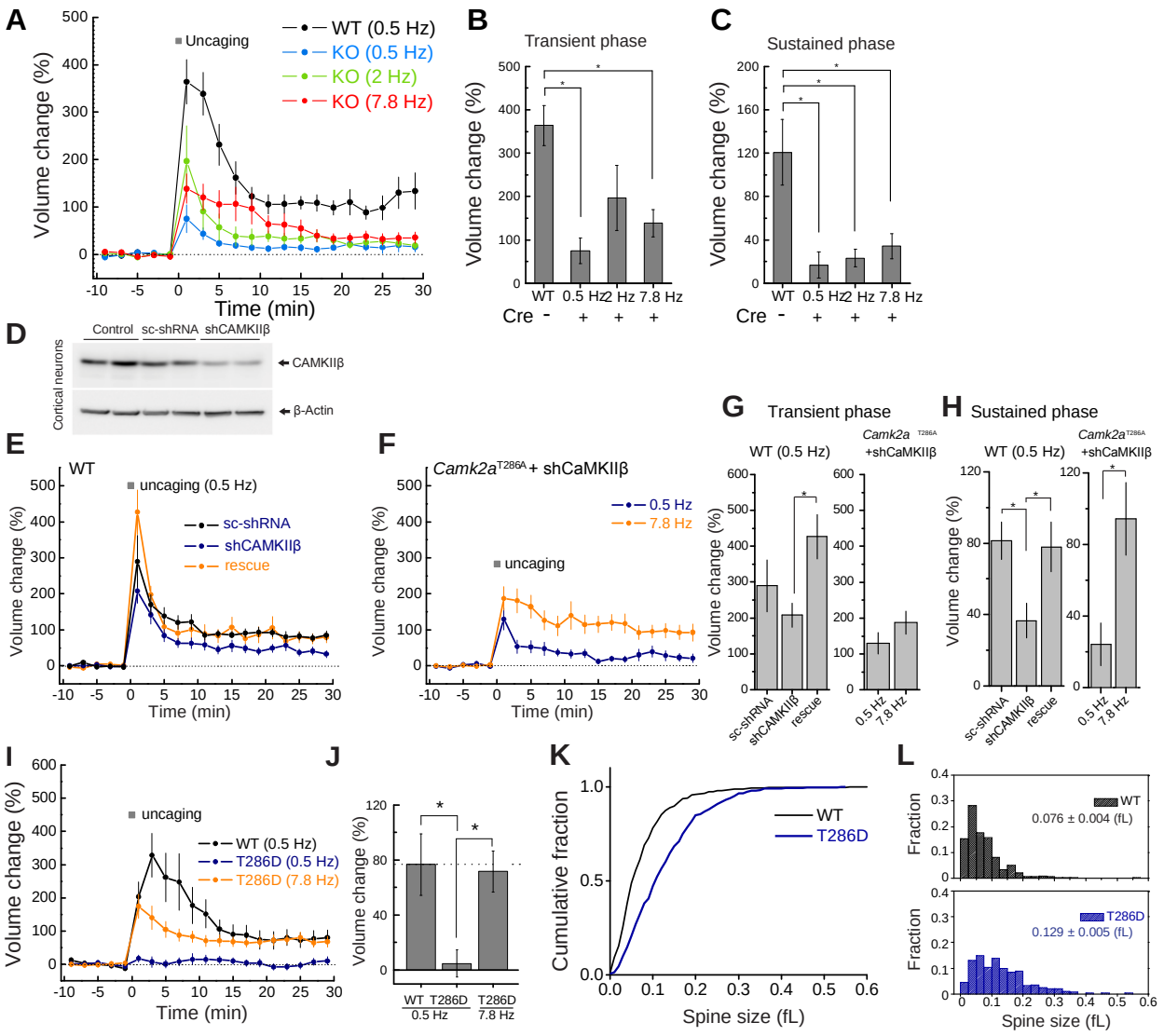

Figure S5. Related to Figure 4

Structural LTP (sLTP) induced by high frequency stimulation

(A) sLTP induced at CA1 neurons from *Camk2a*$^{fl/fl}$ mice with exogenous expression of Cre recombinase. WT group (black): no Cre recombinase, glutamate uncaging at 0.5 Hz for 30 pulses (n = 26 spines/13 neurons). Blue: glutamate uncaging at 0.5 Hz for 30 pulses (n =15 spines/13 neurons). Green: glutamate uncaging at 2 Hz for 120 pulses (n =12 spines/11 neurons). Red: glutamate uncaging at 7.8 Hz for 120 pulses (n =17 spines/12 neurons).

(B-C) Quantification of spine volume change during transient phase (B; peak value recorded at 1 min) and sustained phase (C; averaged over 25-30 min). Spine volume change induced by glutamate uncaging in *Camk2a*$^{fl/fl}$ CA1 neurons was significantly impaired at all frequencies compared to the spine volume change induced by 0.5 Hz glutamate uncaging in the control group (black).

(D) Validation of *Camk2b* shRNA efficiency. Dissociated mouse cortical neuron cultures were infected with Lentivirus encoding shRNA against CaMKIIβ. Endogenous CaMKIIβ level was 30-50% lower in shRNA infected neurons compared to scramble shRNA transfected neurons (n =4 repeats per experimental condition/2 cultures).

(E) sLTP induced at CA1 pyramidal neurons from wild-type littermates of *Camk2a*^{T286A} mice. Black: neurons expressing scramble shRNA (sc-shRNA; n = 16 spines/6 neurons). Blue: neurons expressing shRNA against

Camk2b (shCaMKIIβ; n = 24 spines/7 neurons). Orange: neurons expressing shRNA against *Camk2b* together with shRNA resistant CaMKIIβ from rats (rescue; n = 23 spines/8 neurons).

(F) sLTP induced at CA1 pyramidal neurons from *Camk2a*^{T286A} mice with the expression of *Camk2b* shRNA. Blue: glutamate uncaging at 0.5 Hz for 30 pulses (n = 16 spines/9 neurons). Orange: glutamate uncaging at 7.8 Hz for 120 pulses (n =18 spines/8 neurons).

(G) Quantification of spine volume change during the transient phase (1 min) in (E) and (F) respectively.

(H) Quantification of spine volume change during the sustained phase (averaged over 25-30 min) in (E) and (F) respectively.

(I) sLTP in neurons expressing Camuiα^{T286D}. Number of samples (spines/neurons) is 10/6 for T286D induced at 0.5 Hz (blue), 13/5 for WT induced at 0.5 Hz (black), and 14/8 for T286D induced at 7.8 Hz (yellow).

(J) Quantifications of spine volume changes during the sustained phase (I; averaged over 25-30 min).

(K) Cumulative plots of spine volume in neurons expressing Camuiα^{T286D} (blue) and Camuiα^{WT} (black).

(L) Histograms of spine volume in neurons expressing Camuiα^{T286D} (blue) and Camuiα^{WT} (black). Average spine volume is 0.076 ± 0.004 fL for Camuiα^{WT} expressing neurons (n = 318 spines/3 neurons), and 0.129 ± 0.005 fL for Camuiα^{T286D} expressing neurons (n = 283 spines/3 neurons).

Asterisks denote statistical significance ($p < 0.05$; ANOVA followed by *post hoc* Bonferroni test). All data are shown in mean ± s.e.m.

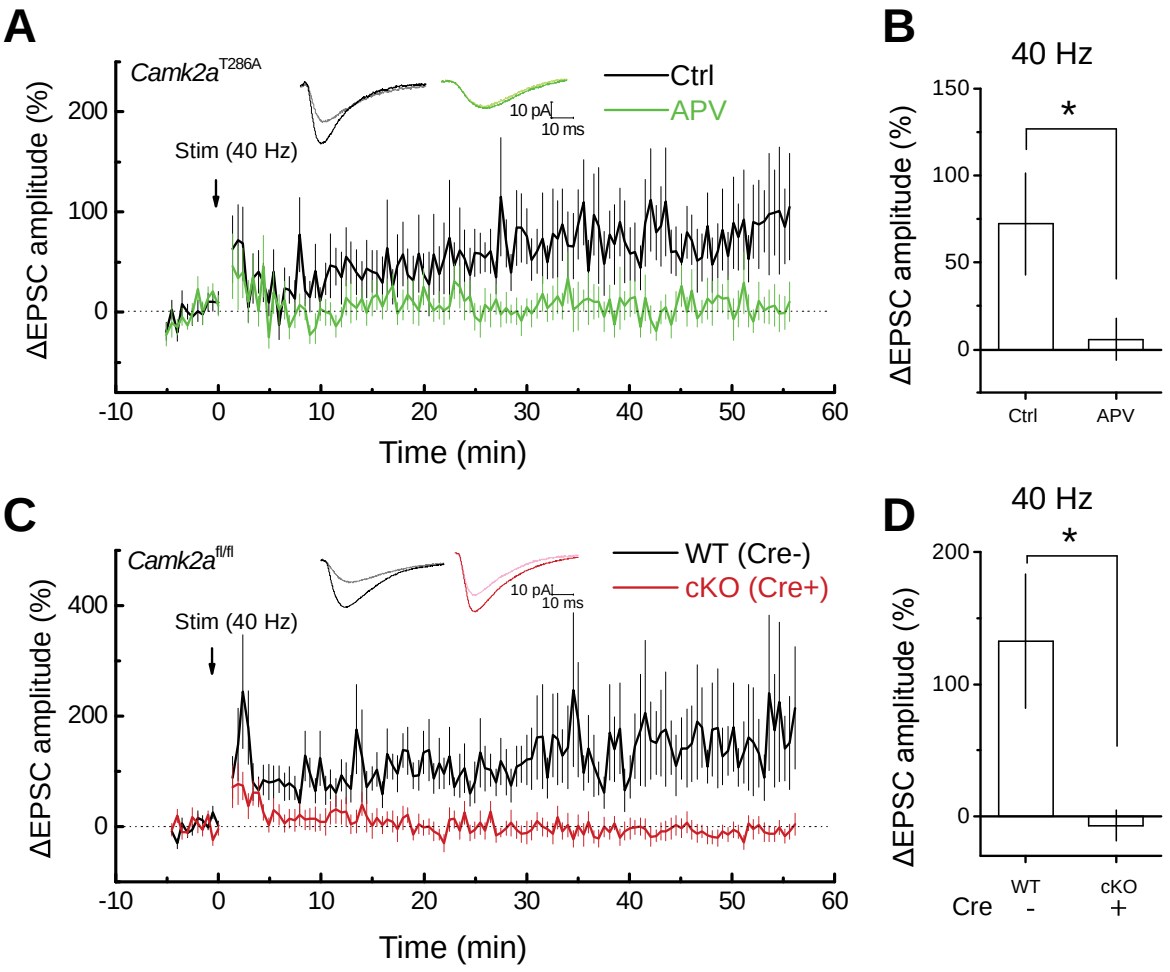

Figure S6. Related to Figure 4

Electrophysiological LTP induced by high frequency stimulation (40 Hz) paired with postsynaptic depolarization

(A) Whole-cell patch-clamp recording of LTP induced at the Schaffer collateral in CA1 neurons from *Camk2a*^{T286A} homozygous mice in the presence and the absence of APV. LTP is induced by presynaptic stimulations at 40 Hz for 15 s with postsynaptic depolarization to 0 mV. Number of neurons is 20 for T286A, 12 for APV. Typical EPSC traces before (-10–0 min) and after (26-56 min) are presented on top of the panel.

(B) Quantification of EPSC potentiation averaged over 26–56 min in A.

(C) Whole-cell patch-clamp recording of LTP induced at the Schaffer collateral in CA1 neurons from *Camk2a*$^{fl/fl}$ mice infected with AAV1.CAG.EGFP (Cre-), and infected with AAV1.CAG.Flex.tdTomato/AAV1.hSyn.Cre (Cre+). LTP is induced by presynaptic stimulations at 40 Hz for 15 s with postsynaptic depolarization to 0 mV. Number of neurons is 11 and 13 for Cre+ and Cre-. Typical EPSC traces before (-5–0 min) and after (26–56 min) are presented on top of the panel.

(D) Quantification of EPSC potentiation averaged over 26-56 min in C. Asterisks denote the statistical significance ($p < 0.05$; ANOVA followed by *post hoc* Bonferroni test). All data are shown in mean ± s.e.m.